Cloning of Subtilisin E Gene from *Bacillus subtilis* into an *E. coli-S. cerevisiae* shuttle vector

Introduction

In the beginning we received the PHY300PKL plasmid, which is designed for expression if Subtilisin E in Bacillus. We chose a shuttle vector carrying the URA gene and consequently an uracil deficient *Saccharomyces cerevisiae* strand. The vector has the secretion tag MF alpha to make harvesting of produced protein more convenient. After cloning the Subtilisin E gene into the vector and amplifying it in *Escherichia coli* we transformed it in *S. cerevisiae* for testing of expression. A scheme of the cloning can be seen below:

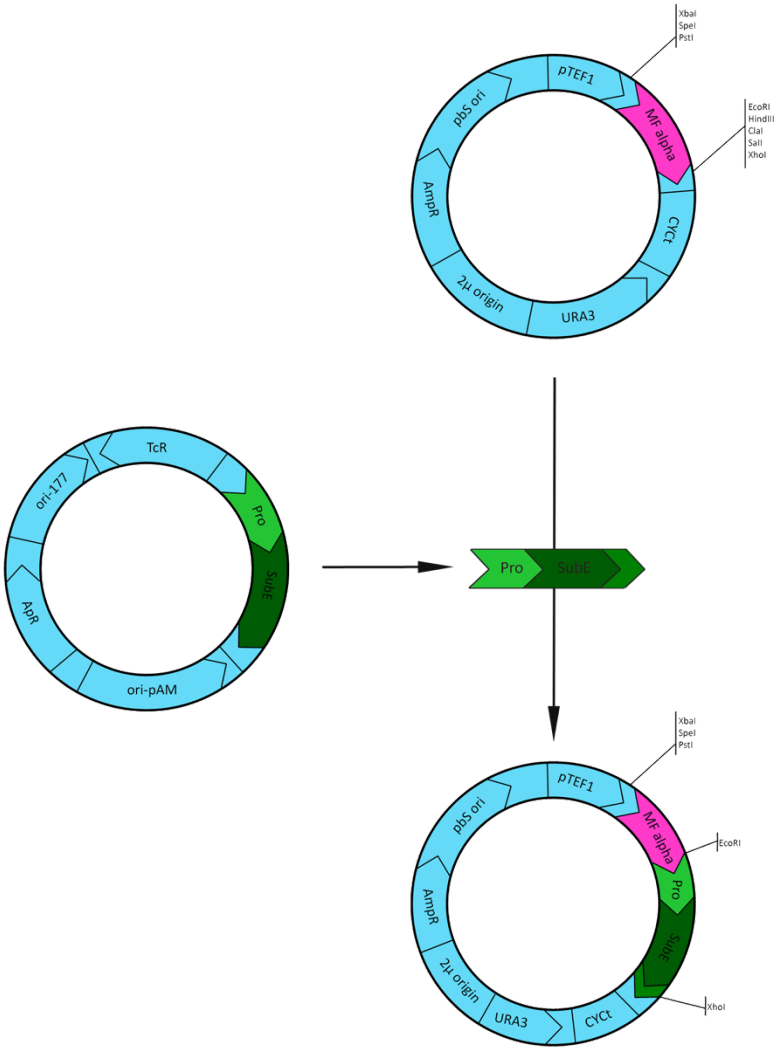

Figure 1: cloning Subtilisin E into pTEF-MF, vector a left side: original PHY300PKL with Subtilisin E gene

All protocols we used can be seen under http://2016.igem.org/Team:Aachen/Lab/Protocols

For all agarose gels we used 1% agarose and 1kb Thermo scientific ladder 08:

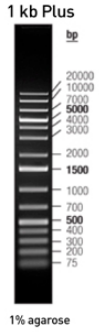

Figure 2: 1kb Thermo scientific ladder

Day 1

Amplification of Subtilisin E gene

We amplified the Subtilisin E gene of the Plasmid PHY300PKL with YP0001 and YP0003 via PCR.

We used 50 ng of plasmid per 50 µl of reaction volume in ten reaction tubes. The annealing temperature was chosen as gradient between 51.5 and 70°C

columns:

1. 51.5°C
2. 53.4°C
3. 55.7°C
4. 58.3°C
5. 61°C
6. 63.7°C
7. 66.1°C
8. 68.0°C
9. 69.4°C
10. 70°C

cycles:

ID	98°C	1 min	
D	98°C	20 sec	
A	50-70°C	30 sec	x24
E	72°C	45 sec	
FE	72°C	3 min	
S	8°C	storage	

Agarose gel

We made an agarose gel to see our PCR product with a size of 1094 bp and run it in a gel electrophoresis. Only columns 6 and 7 showed the insert of the right length.

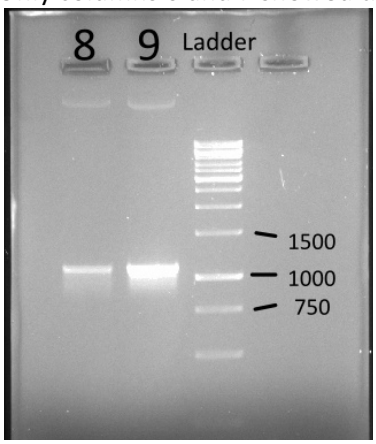

Figure 3: Agarose gel; 8: PCR product of column 6; 9: PCR product of column 7

The size of the insert is right.

PCR clean up

We made a PCR clean-up of the samples.

Nanodrop

We used nanodrop to measure the concentrations of the PCR product after the PCR clean up:

1. 90.3 ng/µl
2. 133.9 ng/µl

Digestion

We collected the two samples in one tube and double digested it with EcoRI and XhoI. We also digested the pTEF-MF empty vector with the same enzymes.

Dephosphorylation

We dephosphorylated the cut pTEF-MF.

PCR Clean Up

After the dephosphorylation we made a PCR clean up of the insert and backbone.

Day 2

Ligation of Subtilisin E and pTEF-MF

Using the the cut samples of yesterday we ligated the Subtilisin E gene into the pTEF-MF vector.

Transformation of pTEF-MF with Subtilisin E into *E. coli* DH5alpha

2 µl of the ligation mixture were transformed into E. coli DH5alpha. The cells were plated on LB+ ampicillin plates each 200 and 800µl. One plate with untransformed cells was made as negative control. Positive control is missing.

Day 3

Results of Transformation

The transformation was successful. The negative control did not grow, so the concentration of antibiotics in the plates was good.

Colony PCR and Overnights

We made a colony PCR with PEG of five of the colonies to see if the colonies have the desired Subtilisin E insert. The conditions were chosen as in columns 6+7 of the amplification of Subtilisin E gene of day 1.

Agarose Gel

We made an agarose gel of the PCR products and loaded the PCR product of the amplification of Subtilisin E as control. The vector in clones 4 and 5 contains an insert.

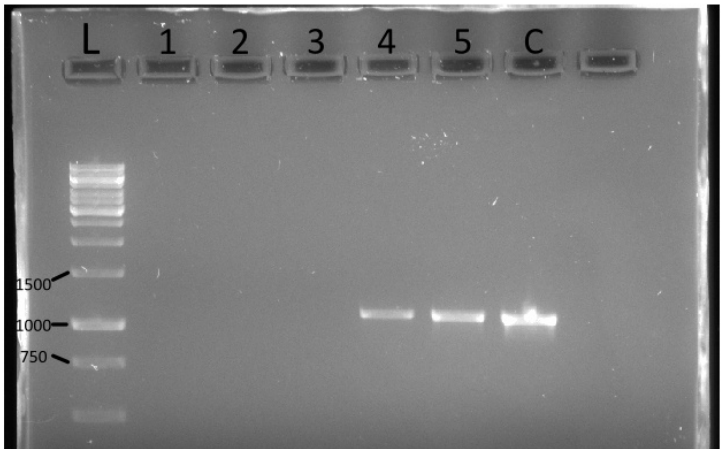

Figure 4: agarose gel; L: ladder; 1: PCR product of colony 1; 2: PCR product of colony 2; 3: PCR product of colony 3; 4: PCR product of colony 4; 5: PCR product of colony 5; C: control: PCR product of day 1 Subtilisin E amplification

Day 4

Cryo Cultures

We made cryo cultures of the overnights we made during the colony PCR yesterday of clone 4 and 5.

Plasmid Preparation

We isolated the plasmids from the rest of the overnight culture of clone 4 and 5.

Digestion + Agarose Gel

To see if the restriction sites we added with our designed primers YP0001 and YP0003 are working we cut the plasmids with EcoRI and XhoI.

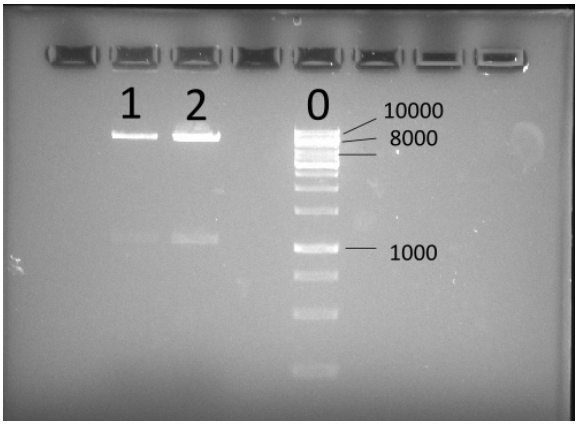

Figure 5: Agarose gel; 1: clone 4; 2: clone 5; 0: Ladder

On the gel it is visible that in both clones the restriction sites are intact.

Sequencing

We sent the isolated plasmids in for sequencing with YP0007 and YP0008.

Day 5

Sequencing Results

The sequencing showed different sequences from the same primer We guess that is so the medium of the overnights was probably contaminated.

Dilution Plating

We did a dilution plating on LB+Amp medium to get get rid of the contaminations.

Day 6

Overnights

We picked two colonies of each plate (clone 4 and 5) and made overnights of the dilution plating in LB + Amp medium for plasmid isolation and sequencing tomorrow.

Day 7

Plasmid Preparation

We isolated the plasmids of the overnights of the dilution plating.

Nanodrop

A nanodrop was made from the isolated plasmids

clone 6: 445.0 ng/µl
clone 7: 501.1 ng/µl
clone 8: 898.5 ng/µl

Test Restriction
We digested the isolated plasmids with EcorI and XhoI

Agarose Gel
We made an agarose gel and loaded the test restriction's samples. The expected insert size is about 1100 bp.

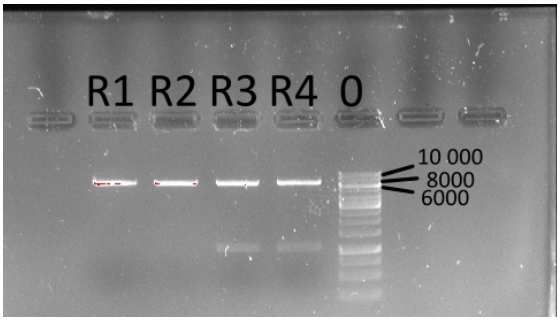

Figure 6: Agarose gel; R1: clone 6; R2: clone 7; R3: clone 8; R4: clone 9; 0: Ladder

Clone 8 and 9 showed the right insert with a size of 1100 bp.

Sequencing
We sent the isolated plasmids again for sequencing with YP0008 and YP0009. As with primer YP0007 we could not cover the complete gene of Subtilisin E with signal peptide

Day 8

Sequencing Results
Sequencing results of clone 3 and 4 were good.

Transformation in *S. Cerevisiae*
We transformed the plasmids of clone 3 and 4 into S. cerevisiae CENPK2-1D.

Day 11

Transformation Results
pTEF-MF emty vector -> many colonies
pTEF-MF + Subtilisin E -> some colonies
untransformed cells on SD-U medium -> no colonies
The transformation was not very efficient but worked.

Overnights *S. cerevisiae*
We made 10 x 5ml overnights from colonies of the transformation in SD-U + Amp medium.

Day 12

Cryo Cultures

We made cryo cultures of the 10 overnights.

Plasmid Isolation *S. cerevisiae*

We spun down the cells of 3 ml overnight culture in 1,5 ml tubes, discarded the supernatant and added 500µl of A1 buffer before vortexing with small glass beads for 30 minutes, to destroy the cell walls. Then we continued with the usual procedure for plasmid preparation.

Nanodrop

We measured the concentration of the isolated plasmids from yeast via nanodrop:

1. 160.2 ng/µl
2. 221.8 ng/µl
3. 65.2 ng/µl
4. 90 ng/µl
5. 70.4 ng/µl
6. 77.3 ng/µl
7. 121.8 ng/µl
8. 145.3 ng/µl
9. 221 ng/µl
10. 155.8 ng/µl

Agarose Gel

We run an agarose gel to see if we can see the isolated plasmids from *S. cerevisiae* at the right size.

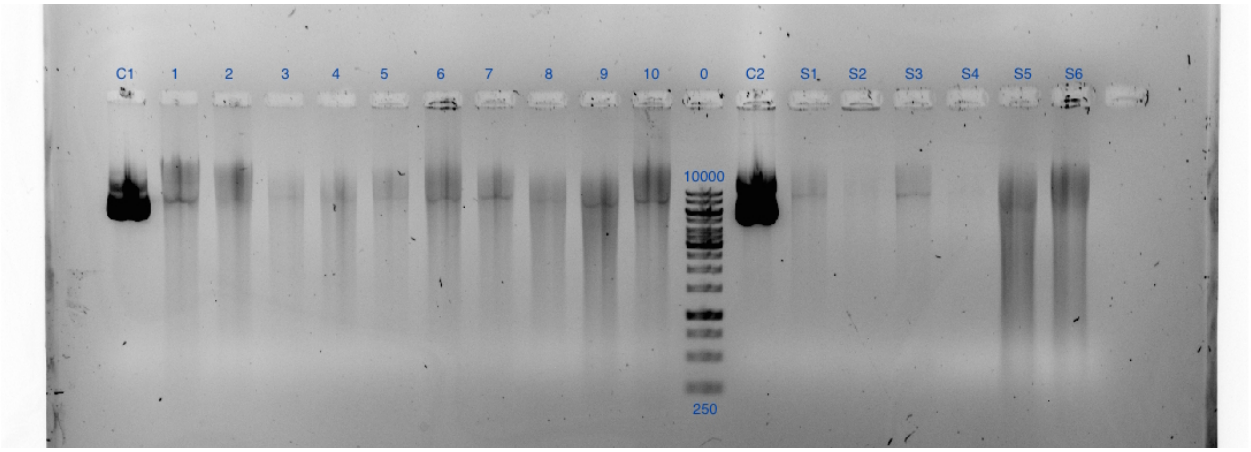

Figure 7: Agarose gel; C1: positive control pTEF-MF + SubE; 1-10: isolated plasmids from S. cerevisiae overnights; 0: ladder; (C2-S6 is irrelevant for this section)

There were no clear bands visible on agarose gel. It is not possible to see *S. cerevisiae* plasmids after plasmid preparation direct on an agarose gel.

Transformation in *E. coli* Dh5alpha

We transformed two of the isolated plasmids from yeast again into *E. coli* DH5alpha to purify them and see them again on an agarose gel.

Day 13

Transformation Results
All transformation yielded colonies.

Overnights *E. coli* DH5alpha
We made two overnights of the transformation plates in 5ml LB + Amp medium

Day 14

Plasmid Isolation
We isolated the plasmids of the DH5alpha overnights pTEF-MF with Subtilisin E.

Restriction
We cut the isolated plasmids and as a control the isolated plasmid pTEF-MF + Subtilsin E from DH5alpha with EcoRI.

Agarose Gel
We ran the cut plasmids on an agarose gel.

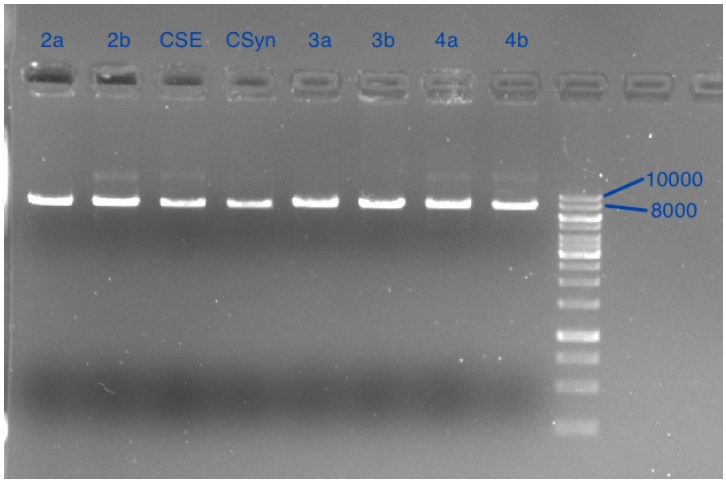

Figure 8: Agarose gel; 2a: clone a pTEF-MF SubE; 2b: clone b pTEF-MF SubE; CSE: pTEF-MF SubE control; (Csyn-4b is not relevant in this section)

It is visible that all the plasmids have the right size.

Sequencing
We send the plasmids for sequencing of insert with primers YP007, YP008 and YP009.

Result

The sequencing results showed a perfect sequence of secretion tag and Subtilisin E gene.

After Day 14 our expression system was completely ready and testable. See the next section: test expression.